

## TITLE OF THE INVENTION

METHOD FOR EFFICIENT TRANSPORT OF SMALL LIQUID  
VOLUMES TO, FROM OR WITHIN MICROFLUIDIC DEVICES

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## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. Provisional  
Application No. 60/528,410 filed December 10, 2003 entitled,  
METHOD FOR EFFICIENT TRANSPORT OF SMALL LIQUID VOLUMES IN  
10 MICROFLUIDIC DEVICES, the whole of which is hereby incorporated by  
reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR  
DEVELOPMENT

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N/A

## BACKGROUND OF THE INVENTION

In the last 50 years, and especially in the last decade,  
20 there has been a remarkable trend towards both the automation and  
the miniaturization of chemical analysis and electromechanical  
systems. The limits of detection of primary analytical methods  
have improved by many quantum leaps. Mass spectrometry can detect  
attomoles of sample using nanospray methods. Nuclear Magnetic  
25 Resonance (NMR) can now detect pmoles of analyte using 5 nL  
microcoil probes (Olson, 1995), a 500-fold improvement over 1980's  
technology. Capillary electrophoresis with laser-induced  
fluorescence detection (CE-LIF) can detect zeptomoles of analyte  
in a volume of picoliters. The tremendous sensitivity of these  
30 microscale analytical technologies, however, is useless without  
the ability to efficiently load and deliver the appropriate  
microscale samples. For example, capillary electrophoresis  
analysis generally requires providing several microliters of

sample, from which a few nanoliters is drawn. Loading a 1  $\mu$ L microcoil NMR probe requires filling a 10  $\mu$ L dead volume. On microfluidic chips, samples typically are introduced to fill entire channels, of which only a small segment may occupy a region of detection or be injected into a separation channel.

An obvious alternative would be to supply small samples and drive them through the conduit either with air or with clean solvent. However, in pressure-driven liquid flow, a sample originating as a short volume segment of a conduit will disperse into a larger volume, with concomitant dilution, proportionally to the volume through which it is moved: the boundary layer at the conduit wall is immobile; however, flow at the center of the conduit is rapid. Although dispersion of small concentrated samples can be significant even within the few-cm distances of a microfluidic chip, the problem is most vividly defined and discussed in the example of flow-NMR, where samples must be transported over distances of several meters.

NMR is a very information-rich spectroscopy, well-established for confirming the structure and purity of newly synthesized compounds or isolated natural products. It has also proven valuable in metabonomics, using pattern recognition software to analyze large numbers of complex spectra. However, the low sensitivity of NMR (1000-fold less than mass spectrometry) is problematic, particularly in LC-NMR where acquisition time is limited and compounds of interest may be a small fraction of the permissible column load. NMR sensitivity has been improved modestly (2-4-fold) using higher field magnets and cryogenically-cooled electronics (cryoprobes). For mass-limited samples, microcoil NMR probes offer up to a 500-fold sensitivity increase (Olson, 1995), however, efficiently loading microcoil probes is a challenge (Kautz, 2001). The detection cell has a volume of 30 nL to 1  $\mu$ L is recessed 50 cm or more up the narrow bore of the NMR magnet. In contrast to conventional NMR probes, the microcoil

probe's axis is oriented transverse to the magnet bore so sample tubes cannot be inserted without removing the probe, and consequently microcoil probes are generally implemented as a flow cell. An additional complication is that any motorized equipment must be located outside the magnet's fringe field, necessitating an additional 1-10 meters of capillary tubing, depending on the magnet's fringe field. The current commercial offering is a compromise with these limitations, using the smallest feasible transfer capillaries to fill a relatively large flowcell. But the challenge of filling a 1  $\mu$ L observed volume in a 5  $\mu$ L flow cell through several meters of 50 micron capillary tubing ( 2  $\mu$ L/meter) has severely limited microcoil NMR's sensitivity in practice.

The two traditional approaches to flow-NMR (Keifer, 2003a) are direct-injection NMR (Keifer, 2000) and flow-injection analysis-NMR (Keifer, 2003b). The methods differ in how they optimize the necessary steps of clearing, washing and reloading the NMR probe flow cell through the 2-5 meter transfer line while avoiding sample dilution in the dead volumes of the transfer line and NMR probe flow cell. In direct injection NMR, samples are injected into an empty (air-filled) flow cell through a 100  $\mu$ m i.d. or larger transfer line. Samples can be injected relatively quickly without dilution; however, the percentage of the injected sample that ultimately resides within the NMR coil observed volume during spectral analysis, is low. The need for a wash cycle to reduce sample-to-sample carryover to <1% increases the sample change time. Because it is not feasible to flush 50 micron capillaries longer than 1 meter with air, and larger capillaries have a prohibitively large volume, direct injection methods have only been implemented on microcoil probes manually. Working at the closest approach to the magnet bore, it is possible to fill the flow cell using 8  $\mu$ L samples.

In flow injection NMR, the flow lines are maintained filled with solvent. Samples are introduced by means of a sample loop

valve and delivered to the probe by a liquid chromatographic pump. Because the sample disperses into the carrier solvent during transfer, the final analyte concentration in the NMR coil depends on the sample volume, flow rate, and system dead volume. Sharp  
5 gradients of analyte concentration near the NMR coil immediately after injection can cause poor line shape, and an equilibration time of 1-2 min may be required for line shape to sharpen as the analyte diffuses throughout the flow cell. Because the effects of these gradients are more pronounced for dissimilar solvents, the  
10 same solvent must be used for both the carrier and sample preparation.

FIA-NMR methods are applicable to microcoil probes, and a high-throughput FIA-NMR method using a commercial microcoil probe with a microfluidic sample loader (Olson, 2004) has been  
15 introduced. This method requires 10  $\mu$ L of sample to deliver at full concentration, or dilutes smaller samples to a dead volume of 10  $\mu$ L in the course of loading. 50  $\mu$ L of deuterated solvent was also required per sample to reduce carryover below 1%.

Another approach is segmented flow, in which an immiscible  
20 fluid is used to push a small sample as a bolus or "plug" through the fluidic conduit. This approach appears to offer several advantages. Smaller samples could be used, so sample consumption would be lower. Samples would not be diluted, so NMR acquisition time would be faster. Samples could be more accurately positioned  
25 in the detection cell, so setup would be straightforward, faster and provide better sensitivity. There would be no "equilibration time" required for lineshape to improve after injection. And for high-throughput operation, a queue of samples could be quickly advanced a short distance, rather than having each new sample  
30 delivered the entire conduit distance. While the stability of segmented plugs in the 3-mm vertical flow cells of conventional saddle coil LC/NMR probes is problematic, several preliminary findings with segmented plugs in microcoil NMR probes appear

promising. Segmented flow has historically been implemented in clinical analyses and has recently been demonstrated in a microfluidic chip (Ramsey, 2003).

In work on the optimal sizes of microcoil probes, it was shown that samples sandwiched on both sides by the immiscible fluorocarbon fluid FC 43 could be much smaller than samples sandwiched between air bubbles without degradation of the NMR line shape: only twice the coil size instead of 7 times (Behnia, 1998). The utility of this fluorocarbon bracketing was demonstrated in obtaining spectra from the 500-ng eluate of a single solid-phase synthesis bead (Lacey, 2001).

However, substantial challenges remain in putting this approach into practice (Macnaughtan, 2003). Sample plugs are frequently lost or degraded in a variety of ways. Principally, moving sample plugs leave a film of solvent on the wall of the conduit, e.g., capillary, and this film can consume about 2  $\mu$ L of sample per meter of movement, which is completely prohibitive. All of this lost material can mix with subsequent plugs, resulting in high carryover (Patton et al., 1997). Plugs also have tended either to acquire large discrete breaks in the middle ("fragmentation") or to form many small breaks ("frothing") at their ends. Both of these effects increased with increasing capillary size, where the outward pressure of the curved surface of the plug was insufficient to hold the plug against the conduit wall in capillaries over 300  $\mu$ m diameter. Improvements in these techniques would be greatly appreciated.

#### BRIEF SUMMARY OF THE INVENTION

The system and method of the invention provide solutions to the problems identified above. This invention is directed to moving small samples through conduits, e.g., the capillary channels or tubing of a microfluidic device, without dilution of

the sample or loss of sample to the capillary wall. The transported sample is a small volume of liquid, for example a solution of an analyte for chemical analysis.

5 In the preferred embodiment of the method of the invention, an aliquot of a liquid that is not miscible with the sample, denominated an "immiscible carrier liquid," is first introduced into a conduit through a microfluidic device. When an aliquot of the sample is subsequently introduced into the conduit, the sample forms a segment or "plug" in the microfluidic channel or  
10 capillary, following the carrier liquid. The carrier liquid is pumped or otherwise caused to flow through the channel, and the sample is carried from one location to another through the microfluidic channels without dilution or dispersion into the immiscible carrier liquid. The interior wall of the conduit  
15 (channel) is covalently coated with a suitable coating, so that the carrier liquid wets the conduit wall preferentially to the sample solvent. A film of the carrier liquid will then be retained on the channel wall as the sample plug is moved passed, so that the moving sample plug will not contact the conduit wall.  
20 This avoids losses of analyte either by binding of analyte molecules to the conduit wall or by bulk loss of sample as a film on the conduit wall.

Preferably, the microfluidic device is part of a conduit system, with attached tubing to transport samples onto and off of  
25 the device. Small liquid samples may thus be transported long distances through microfluidic plumbing and through such microdevices with very low losses, and at relatively high speed. As a specific example, if the immiscible carrier liquid is a fluorocarbon (FC), and the channel surface is fluorine-rich, the  
30 carrier liquid will wet the channel wall preferentially to both aqueous and organic (hydrophobic and hydrophilic) solvent samples. The desired effect may be obtained either by making a portion of the system, e.g., the attached tubing, of a fluorine-rich material

such as a teflon (PTFE, ETFE, FEP, NGFP, etc.), or a channel wall, e.g., in the microdevice, may be coated with a fluorine-rich layer such as a fluoroalkyl silane coating on glass, silica, or plastic.

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#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

**Figure 1** shows the efficacy of the present invention through several photographs of the delivery of a visible dye sample into a glass flow cell under various conditions. The test flow cell is identical in material composition to the microcoil NMR probe flow cell of the microcoil NMR probe used herein, and sample was delivered through 1 meter of 50 micron fused silica capillary. Panel A shows that 1  $\mu$ L of dyed DMSO injected in a flowstream of DMSO by the conventional FIA-NMR method has dispersed throughout the flowcell. Panel B shows 1  $\mu$ L of dyed DMSO when injected using the zero-dispersion segmented flow approach of this invention. (The flowcell and inlet capillary in these two photographs have been coated with dichlorodimethyl silane to make their surfaces less hydrophilic and more wettable by the carrier liquid).

**Figure 2:** Comparison of unfavorable, favorable, and ideal conditions for zero-dispersion segmented flow transport of sample plugs. In these photos, the sample is DMSO with 10 mg/mL of the blue dye methyl green and the carrier liquid is the colorless fluorocarbon FC-43. In uncoated fused silica capillary (top) the DMSO plugs are hourglass-shaped, as the sample wets the bare silica wall preferentially over the fluorocarbon. The smear at left is coalesced from film of DMSO retained on wall as the plug was moved into its current position. DMSO plugs in teflon are

sausage-shaped; the tangential contact angle of the FC 43 with the wall indicates the wall is wetted by fluorocarbon even in the presence of a stationary sample plug. Fused silica capillaries coated with the fluoroalkyl silane perfluorooctyl silane (PFOA) also favor wetting by fluorocarbon; moving DMSO plugs do not leave film on the wall, but can contact the wall if left stationary. All capillaries shown are 200  $\mu\text{m}$  i.d.; the fused silica are 360  $\mu\text{m}$  o.d., the Teflon capillary is 400  $\mu\text{m}$  o.d.

**Figure 3.** Demonstration of the improvement in the sensitivity of NMR analysis using the present invention. Five NMR spectra are shown which were acquired from loading of the indicated volumes of a standard sample, 0.3 mg/mL beta-glucoside in  $\text{D}_2\text{O}$ . In the bottom spectrum, the NMR flow cell was completely and uniformly filled with the sample, similar to the conventional direct-injection method. As the volume of the sample plug injected is decreased, there is no decrease in NMR sensitivity, reflecting that the smaller samples are not being diluted yet still fill the observed volume of detection. Only when the sample plug is half the size of the NMR observed volume (top spectrum) is there a decrease in signal strength, because only half as many analyte molecules are being detected. The ability to acquire the same quality of spectrum with a sample volume of 1  $\mu\text{L}$  where the conventional art requires 5  $\mu\text{L}$  permits mass limited samples to be loaded at five times the concentration, increasing sensitivity in practice by a factor of five. For reference, it is worth noting that cryogenically-cooled NMR probes are a commercial product which offers a factor of 4 sensitivity enhancement at a cost of approximately \$200,000,

**Figure 4. (A)** Schematic diagram of an implementation of the present invention for high-throughput micro-NMR analysis. Samples (in DMSO, drawn in black) were loaded as a train of "plugs" separated and carried by an immiscible liquid (FC 43, drawn light gray). Samples were changed by advancing the queue until the next



sample plug was centered in the NMR coil. Wash plugs of solvent (white) were included to reduce carryover in non-ideal components. At the higher linear velocity in the narrow inlet and outlet capillaries, the plugs are separated from the capillary wall by a layer of the carrier liquid. At the slower linear velocity in the flow cell, the plugs may contact the surface.

(B) photograph of a sample plug (dyed DMSO) recovered after automated withdrawal from a vial, delivery through a 2 meter transfer line, and passage through a commercial microcoil NMR probe which included 2 meters of 50 micron fused silica capillary in addition to the NMR flow cell.

**Figure 5.** Apparatus for segmented flow analysis NMR. In the center is the sample loop valve of the Protasis HTSL-1100 sample loader. The illustrated "deliver" position placed the sample loop in line between the sample loader pump and the transfer line connected to the NMR probe. In the "fill" position, the sample handler syringe drew sample plugs into the loop via a 200- $\mu$ m-i.d. capillary threaded through the sample handler needle. The sample plugs were formed by alternately drawing samples in DMSO, the immiscible fluorocarbon FC 43, and wash plugs of clean solvent. The transfer line to the probe was 3 m long (43  $\mu$ L). D Kautz et al. *Journal of Combinatorial Chemistry* high-throughput NMR,

**Figure 6.** NMR spectra of a model compound library acquired using segmented flow analysis NMR. The average throughput was 1.5 min/spectrum, using 2  $\mu$ L of 30 mM analyte. Each spectrum was the sum of 16 1-s transients. Compounds: uracil (A), reserpine (B), erythromycin (C), chlorpromazine (D), tolbutamide (E), indomethacin (F), haloperidol (G), 4-acetamidophenol (H), indapamide (I), prilocaine (J), phenylbutazone (K), and brucine (L).

**Figure 7** shows an embodiment where the invention is used for collecting and transporting samples of materials purified and isolated by microseparations. Panel A shows a separation channel, which may be electrophoresis or liquid chromatography. A band of analyte is approaching a side-channel to be used for sample recovery. The side channel intersects a third channel, which has a favorable wall material for zero-dispersion segmented flow and is filled with an immiscible carrier (B) When the analyte band arrives at the side-channel it is drawn in. (C) The withdrawn analyte is transferred as an immiscible plug to subsequent processing or a storage capillary.

**Figure 8:** This embodiment shows a "world-to-chip" interface, whereby laboratory frame samples may be readily applied to, processed in, and recovered from a microfluidic chip. The figure at top (A) is a sectional view through the line AA indicated in the plan view below it (B). As a generic chip, a simple T injector is shown. The samples are made to flow through the chip, and may be recovered. The provided samples may be analyzed in transit, or smaller volumes may be withdrawn from the provided samples, as indicated. While segmentation of samples on microfluidic chips was claimed in (Ramsey, 2003), the present invention offers the improvement of eliminating carryover of aqueous samples on glass or silicon surfaces. It is an obvious extension of the principle to perform a "chip-to-chip" interface, where two microfluidic devices are interconnected by capillary tubing using the principles of this invention, as an alternative to, for example, making a new device with features of the two devices integrated side-by-side to facilitate transferring samples between them.

**Figure 9.** A photo of two water droplets on a 3 cm wide silicon wafer. The right side of the wafer has been coated with perfluorooctyl silane; the left side has merely been cleaned with lye. A 10  $\mu\text{L}$  droplet of water was applied to each side, and smeared over 1  $\text{cm}^2$ . The water is repelled by the PFOS-treated silicon, so it re-forms a sitting drop with a contact angle of nearly 90 degrees. The untreated silicon is wetted, and the resulting water film is not apparent in the photograph.

## DETAILED DESCRIPTION OF THE INVENTION

The purpose of activities leading to the invention was to develop an automated segmented flow NMR method that could increase throughput, could utilize low-mass samples efficiently (with minimal sample loss) and could accept samples from a 96-well plates. The target application was high-throughput NMR analysis of combinatorial chemistry library. The primary goal of the development activity was to achieve the highest possible throughput that yielded spectra of sufficient quality to be interpretable by automated spectral analysis software. Gains in sensitivity and sample utilization were also desirable to detect, and if possible to identify, contaminants at the 5% level. Sample-to-sample carryover had to be below 1%, the high operating cost of deuterated solvent consumption was to be reduced as much as possible, and the method was to be implementable with commercially available instrumentation. These development activities led to the method of the invention, a zero dispersion method of segmented flow analysis, implemented at the microscale level.

As an example, a segmented flow analysis approach to microcoil NMR analysis (SFA-NMR) is illustrated in Fig. 4A, a schematic diagram of a microcoil NMR flow cell 2 and adjoining capillary tubing 4 containing several sample plugs 6 separated by an immiscible carrier fluid 8. One sample plug is centered on the NMR detection coil 10 at the moment flow would be stopped, and a wash plug 12 of clean solvent precedes each sample. Because samples do not disperse into the immiscible carrier, the sample plug volume may be the minimum required to obtain high-resolution spectra (typically twice the NMR coil observed volume). Consequently, because samples are not diluted during transfer to the probe, comparable NMR data quality may be obtained with shorter acquisition times than when using an FIA-NMR method. Additionally, because the concentration is uniform, there is not a

1-2 minute equilibration time after injecting the sample before good lineshape can be obtained. In the segmented flow approach to high throughput, the entire transfer line to the sample handler is filled with a queue of many such sample plugs. The detection cell  
5 is cleared of the old sample, washed, and filled with new sample in rapid succession by advancing the queue through the distance of one sample-to-sample separation. Successful implementation of SFA-NMR requires that sample plugs be moved through several meters of transfer capillary between the sample loader and the NMR probe  
10 without the plugs becoming fragmented or analyte adsorbing onto capillary surfaces.

Zero dispersion segmented flow methods (Patton, 1997) have been demonstrated in larger scale systems and are based on the principle that if the carrier fluid has a favorable contact energy  
15 with the tubing wall, relative to the sample, a film of carrier is maintained between the wall and the sample as sample plugs are moved through the tubing, or conduit (Patton, 1997; Nord, 1984; Adler, 1973). The combination of a fluorocarbon carrier liquid in Teflon™ tubing was recently demonstrated in continuous flow PCR, a  
20 method that is particularly sensitive to carryover (Curcio, 2003). The fluorocarbon FC 43 has been used in building microcoil probes to match the magnetic susceptibility of the copper wire of the coil (Olson, 1995) and has been shown to improve line shape when used to "bracket" small aqueous sample plugs in microcoil NMR  
25 (Behnia, 1998; Webb, 1996). FC 43 also has a relatively high viscosity (2.8 cs) among fluorocarbons, which favors film formation (Nord, 1984).

However, the performance of segmented flow in microfluidic devices tends to be poor. Many preferred conduit materials, such  
30 as glass, fused silica capillary, PEEK tubing or fittings, metal and polypropylene sample tubes or microtiter plates, will retain a film of aqueous or organic solvents and are poorly wetted by fluorocarbon liquids. Treatment of glass with dichlorodimethyl

silane, a conventional well-known hydrophobic coating, is able to abrogate this permanent film retention and permit plugs to be moved slowly, but does not realize favorable for zero dispersion. At the microscale, in conduits below 200 microns, the sample plugs exert an outward force against the conduit walls, and the use of air bubbles to segregate segments cannot be used because bubbles compress to disappearance at the backpressures encountered.

The key to successful practice of the method of the invention for microscale analysis in microfluidic devices, such as the detection cell of an NMR microcoil (or probe) or other microfluidic device such as a microfluidic chip, which are frequently made of glass, fused silica, silicon or other material not easily wettable by fluorocarbon materials, is to ensure the wettability of the conduit wall by applying to the wall a coating that will change its properties. Treatment of glass or silica surfaces with perfluoroalkylsilanes, using covalent bonding methods (Karger, 2002), can transform silica into a favorable material for zero dispersion segmented flow at the microscale level.

For achieving zero dispersion segmented flow in the transport of samples into and out of a microfluidic device, such as a microcoil NMR flow cell, a flexible material that is inherently preferentially wettable by the carrier liquid is connected to the conduit through the device. Teflon™ is a class of exemplary conduit materials to use with a perfluorinated liquid carrier fluid, which is immiscible with either aqueous or most organic solvents used for analytical samples. Teflon™, however, has poor mechanical properties for many microfluidic applications: it is difficult to machine or etch channels in, it is resistant to adhesives, castable Teflons are not resistant to fluorocarbon liquids, and Teflon capillary have poor pressure resistance and are difficult to connect to other components. Thus microfluidic

devices in practice will include many unfavorable materials for segmented flow.

Thus, in exemplary embodiments of the method of the invention, the immiscible carrier may be a fluorocarbon liquid, which is immiscible with both aqueous and organic solvents (apparently all solvents and analytes other than mixed hydrocarbon - fluorocarbon solvents).

The transfer conduit (or tubing) leading into a microfluidic device for practicing the method of the invention is made of a perfluorinated or highly fluorinated material and the conduit within the device is coated with such a material, so that the conduit inner wall surface is preferentially wetted by the fluorocarbon solvent. In this case, a film of the fluorocarbon carrier liquid is maintained between the conduit wall and the sample as it passes, such that the sample for analysis does not contact the conduit wall. This prevents adsorption of the analyte to the wall directly, or bulk loss of the analyte solution to film formation on the channel wall.

The above features permit efficient transfer of small discrete samples for analysis through conduits leading into, out of and within microfluidic devices and show a marked advantage for successfully processing samples compared to uniformly filling the channels; to injecting plugs of sample in clean sample solvent as a carrier fluid; or to using an immiscible organic solvent as carrier liquid without such coating of the conduit wall as in the method of the invention.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

## MATERIALS AND METHODS

**Materials.** Polyimide-clad fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ), and the fluoroalkyl silanes were purchased from United Chemical Technologies (Philadelphia, PA). Fluorocarbon FC 43 was from 3M Corp (St. Paul, MN). Teflon capillary and tubing were obtained from Cole-Parmer (Vernon Hills, IL); PEEK capillary, unions, in-line filters, and adapters were from Upchurch (Oak Harbor, WA). The 96-well PCR plates were obtained from Nunc (Rochester, NY). The compounds of the test library (uracil, reserpine, erythromycin, chlorpromazine, tolbutamide, indomethacin, haloperidol, 4-acetamidophenol, indapamide, prilocaine, phenylbutazone, and brucine) were from Sigma. Deuterated solvents and the reference standards tetramethylsilane (TMS) and trimethylsilylpropionate (TMSP) were purchased from Cambridge Isotope Labs (Andover, MA). All other solvents, buffer salts, and dyes were obtained from Fisher Scientific (Pittsburgh, PA) and were used without further purification.

**Instrumentation.** NMR spectra were acquired on a Varian (Palo Alto, CA) Inova spectrometer with an 11.7-T (500 MHz) actively shielded magnet and a flow NMR package consisting of a Gilson (Middleton, WI) model 215 sample handler and Varian VAST automation software. The Gilson 215 was fitted with a 100- $\mu$ L syringe, and supplemental VAST automation programming (Tcl scripts) was written, as described below. A sample loader, model HTSL-1100, from Protasis Corp (Marlborough, MA) consisted of a sample loop valve, high-pressure pump, and microprocessor controller. It could either be triggered to deliver a specified volume and rate, or it could be controlled through an RS232 serial connection.

The microcoil NMR probe principally used in these studies was built in-house, as previously described (Kautz, 2001; Olson,



1999). Copper wire (7.5 turns of 50  $\mu\text{m}$  wire) was wrapped on a glass flow cell with 660  $\mu\text{m}$  i.d., 920  $\mu\text{m}$  o.d. (see Fig. 4A). The 1.1-mm coil enclosed an observed volume of 0.5  $\mu\text{L}$  of the flow path lumen. The flow cell was ~0.8 cm long, tapering over an additional 1.5 cm on each side to match the inner diameters of 75/360 and 100/360- $\mu\text{m}$  i.d./o.d. fused-silica inlet and outlet capillaries. All but 5 cm of the 75- $\mu\text{m}$  inlet capillary was replaced with 100- $\mu\text{m}$ -i.d. Teflon tubing. The glass and silica elements of the flow path were coated with perfluorooctyl silane (PFOS), as described below. The circuit was singly tuned to a proton frequency of 500 MHz, and spectra were acquired unlocked.

Preliminary data were obtained using a commercial microcoil probe, the  $^1\text{H}$  capLC microflow probe (Olson, 2004) manufactured by Magnetic Resonance Microsensors (MRM, Savoy, IL) and distributed by its parent company, Protasis Corp. This probe had a 1.1- $\mu\text{L}$  observed volume ( $V_{\text{obs}}$ ) in a flow cell volume of ~3.5  $\mu\text{L}$ , with 50- $\mu\text{m}$  fused-silica inlet and outlet capillaries. When this probe was used, all connections in the sample loop and transfer lines were made using Upchurch PEEK unions.

The sample handler and loader were connected to the NMR probe as shown in Fig. 5. Both the needle line and sample loop were 70cm of 200/400- $\mu\text{m}$  i.d./o.d. Teflon capillary; the transfer line to the probe was 2 m of 150/400 Teflon capillary; the probe inlet consisted of 1 m of 100/400 Teflon with a residual 5 cm of 75- $\mu\text{m}$ -i.d. fused silica at the connection to the flow cell. To connect the soft Teflon capillary to valves or unions, 2-cm-long pieces of PFOS silica (see below) were butt-jointed to the Teflon using shrinkwrap tubing. The open triangle indicates a bleed valve to facilitate flushing the sample handler path. The bleed valve connected to the sample handler syringe with 1 m of 500- $\mu\text{m}$ -i.d. 1/16-in-o.d. PEEK tubing, and all remaining connections were made

using 250/360- $\mu$ m i.d./o.d. fused silica. Before each use, the system was flushed with FC 43, freshly degassed under vacuum. Flow rates and system volumes were measured by displacement of a dye plug in a calibrated section of 30-gauge Teflon tubing (~300 $\mu$ m i.d.) capturing the effluent from the probe outlet capillary.

**PFOS Silica.** Probes were internally coated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (perfluorooctylsilane, PFOS) monolayer. Surfaces were first activated by washing with 1 N NaOH for 1 h, followed by sequential rinses with water, acetone, and chloroform. A fresh 5% (v/v) solution of PFOS in chloroform was flushed slowly through the probe for 1 h, then the probe was rinsed sequentially with chloroform, acetone, and 10% D<sub>2</sub>O in acetone. Surfaces were exhaustively dried with an acetone rinse followed by air flow overnight, then the probe was stored filled with FC 43.

Fused-silica capillaries were coated with a thicker PFOS gel layer using the trimethoxy form of PFOS according to the manufacturer's protocol (United Chemical Technologies). In brief, fused-silica capillaries were washed with peroxide/sulfuric acid, then activated with 1 N NaOH overnight. A solution was first prepared of 95% methanol and 5% water, with acetic acid added to an apparent pH meter reading of 5. Subsequently, 4% (v/v) tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trimethoxysilane was added with vigorous stirring and allowed to react for 1/2 h before introduction to the capillaries for 16 h at room temperature. Capillaries were annealed for 2 h at 80°C, flushed with the methanol/water/acetate solution, blown dry with helium, then cured and dried at 110°C with helium flow. The capillaries were stored filled with fluorocarbon FC 43 until use.

**Automation.** Automation was controlled using Varian VAST automation programming on the spectrometer host computer (Sparc Ultra 5, Solaris 8, vnmr 6.1C NMR software). NMR acquisition setup

macros were written to (1) automatically detect and position an arriving sample and (2) set up a standard spectrum of a sample (16 scans, 1.05-s acquisition time, 16-Kb points). Four sample handler programs (Tcl scripts) were written to (1) form a train of four samples and hold it in the needle line, (2) draw a train from the needle line into the sample loop, (3) change samples by triggering the sample loader to run until stopped by the autodetection macro, and (4) initialize the sample queue by moving a sample train one-half of the distance from the sample loop to the NMR probe. The sample loader was controlled by means of Unix shell scripts, which could be called from either vnmr macros or tcl scripts.

**Sample Preparation.** The test library of 12 known pharmaceutical entities was prepared as 1-mL aliquots at 30 mM in DMSO- $d_6$  and stored at 4°C. For carryover measurements, standard samples were 4% chloroform- $h$  in DMSO- $d_6$  (standard S1) and 2% acetone- $h_6$ , 5% green food color in DMSO- $d_6$  (standard S2). Ninety-six-well plates were typically alternating columns of S1 and S2, with the third and sixth columns replaced by the 12-member model library. For automated NMR runs, all wells contained 3  $\mu$ L unless otherwise indicated, and plates were covered with adhesive film. Sample plates were placed on 4-mm foam rubber pads on the sample handler racks, carefully positioned under the needle, and taped into position. Two additional vials on the sample handler rack supplied 0.5 mL of FC 43 and 0.1 mL of the wash/autodetection solution, 1% TMSP (60 mM) in DMSO- $d_6$ .

**NMR Spectroscopy.** Sample spectra shown were 16 transients of 16-Kb points, 8000-Hz width, 45° tip angle, auto gain, with no additional relaxation beyond the 1.05-s acquisition time. The spectra were processed by zero-filling to 64-Kb points and Fourier transformed with 1 Hz of exponential line broadening. (Line widths are reported with no line broadening.) Autodetection used single scan spectra with a 60° tip angle, fixed gain, and other

parameters as above; the region from -0.5 to +0.5 ppm was monitored for a peak with S/N > 10 to detect the TMSP in the wash plugs. The COSY spectrum was a magnitude COSY, 128 increments of 16 transients processed with linear prediction in t1 to 512 points, apodized with sinebell-squared matched to acquisition time in both time domains. Total acquisition time was 10 min.

#### EXAMPLE I

##### **Feasibility Studies and Method Development**

Preliminary studies were made, observing the movement of DMSO sample plugs using FC 43 as the carrier fluid in capillaries of several different materials. In the Teflon capillary, FC 43 was the continuous phase: DMSO plugs did not contact the capillary wall and could be moved through several meters with no detectable carryover (<0.1%) or losses at all flow rates tested (0-20  $\mu\text{L}/\text{min}$ ). In plain fused-silica capillary, DMSO was the continuous phase, retention of a DMSO film depleted sample plugs by 2  $\mu\text{L}$  for each meter of movement (in 200- $\mu\text{m}$  capillary). In PFOS-coated silica capillary, neither phase was continuous: a DMSO film was not retained, so sample losses were negligible at modest flow rates (1-10  $\text{L}/\text{min}$ ); however, carryover of minute droplets could occur (10-100 nL/m) if imperfections existed in the coating. It was also found necessary to push the sample train through the NMR probe under positive pressure rather than to pull samples through the system with a syringe or peristaltic pump at the detector outlet, as in a traditional SFA system (Patton, 1997). The flow rate of FC 43 through the microcoil probe with vacuum applied to the outlet capillary was <1  $\mu\text{L}/\text{min}$ ; changing samples in 30 s would require a flow rate on the order of 10  $\mu\text{L}/\text{min}$ , which could be obtained with modest pressures of 150 psi. We therefore pursued a strategy of pulling sample and FC 43 plugs into a sample loop,

then pushing the sample train through the transfer line and microcoil NMR probe by positive displacement.

To facilitate adoption of the method, it was implemented by modifying a conventional microVAST installation. The sample handler and loader were connected with the microcoil NMR probe using Teflon capillary, as shown in Fig. 5. Sample plugs were loaded into the sample loop by drawing consecutive plugs of FC 43, wash solution, FC 43, and sample via a 200- $\mu$ m Teflon capillary threaded through the sample handler needle. The transfer line to the NMR coil held two trains of four samples with a 7- $\mu$ L gap between them. Samples were automatically positioned in the NMR coil by calibrating a delay between initial detection of their NMR signal (FC 43 has negligible  $^1\text{H}$  or  $^2\text{H}$  signal) and stopping the sample loader. The sample handler could operate independently of the NMR spectrometer and sample loader: during the time the sample loop was clearing slowly as four NMR spectra were being acquired from the train in the NMR probe, the sample handler was forming a new sample train from the next four wells of the microtiter plate. To avoid interrupting analysis, this new train was held in the needle line until the sample loop was cleared. Once assembled, the system was calibrated, and flow rates, plug volumes, and automation timing were optimized. A flow rate of 7  $\mu\text{L}/\text{min}$  did not overpressure the 200/400- $\mu\text{m}$  i.d./ o.d. Teflon capillary sample loop. Plugs of FC 43 as small as 0.3  $\mu\text{L}$  were equally as effective as larger plugs in separating DMSO plugs through the probe, as measured by carryover. Plugs of FC 43 of 0.7  $\mu\text{L}$  or larger provided several seconds with no NMR signal during on-flow NMR, which facilitated autodetection. In calibrating the positioning delay after automatic detection, it was found that variability in signal strength among samples caused variation in positioning, so 1% TMSP was added to the wash plugs to provide a consistent signal for detection. The variability of automatic sample plug positioning

was (0.2  $\mu$ L, due primarily to the 1-s intervals used for initial detection. The 2- $\mu$ L sample plugs provided NMR line widths below 1.5 Hz without reshimming over a 0.5- $\mu$ L window, and no equilibration time was required after stopping flow to observe good line shape. For manual injections, sample plugs of 1  $\mu$ L could be shimmed to routine probe specification of 1.2 Hz. The method of drawing samples directly into segmented plugs was able to recover 2.0  $\mu$ L of 2.5  $\mu$ L deposited into the wells of 96-well plates. Manual recovery of 2.0  $\mu$ L from a PFOS-coated vial using PFOS-silica capillary was also possible. The ability to accurately position samples within the detection volume based on the distinct leading edge was another significant advantage over FIA methods, where the optimum position was easily missed.

## EXAMPLE II

### Performance of the Automated SFA-NMR System

The performance of the system was evaluated by loading samples and acquiring spectra from 96-well plates with 3  $\mu$ L/well of test library compounds (30 mM in DMSO- $d_6$ ) interspersed with standards for assessing carryover and line shape. Automated analysis completed in 2.5 hr/plate, plus 24 min to initialize the queue in which four trains were drawn and two were injected. This initialization time is reported separately because it applied to the first plate but not to subsequent plates of continuous high-throughput operation. Spectra were output at rates of 1/min along each train of four samples. The sample change and wash was completed in 35 s, NMR acquisition was set to 16 s, and the automation software required 10 s of execution and dead time. Sustainable throughput was 1.5 min/sample due to the time required to draw a new train from the needle line into the sample loop and to advance the queue through the gap between trains (105s).

Spectra of the 12 test library compounds are presented in Fig. 6. The sensitivity of all spectra was sufficient to unambiguously confirm their structures. The weakest signals of these 12 compounds were the multiplet at 5 ppm in reserpine (spectrum A) and the 18-Hz-wide amide of tolbutamide at 6.2 ppm in Fig. 6E, both with S/N=15. The intensity of most signals were within the range of 130 and 23. Thus, even with the moderate sensitivity of this home-built probe, it should be possible to identify compounds at 3-fold lower concentration (10 mM) or to detect impurities at the 5% level. For example, minor resonances visible in spectrum K (phenylbutazone) at 6.8 ppm were 13% of the net aromatic intensity.

Compound identification may at times require advanced 2D spectra, or it may be desirable to analyze samples in protonated solvents requiring solvent suppression. Consequently, care was taken in programming the method so that existing macros for setting up established methods could be inserted, such as for gradient shimming, scout-scan solvent suppression, or 2D spectra. For example, a macro to acquire a magnitude COSY spectrum was added as a single line to one automation queue as described in Kautz, 2001. This flexibility was made a priority in development in order to enable 2D spectra to be acquired in a data-dependent manner, that is, to acquire a COSY or TOCSY spectrum if automated analysis of a 1D spectrum fails to confirm an expected product.

Importantly, the analyte in immiscible sample plugs was not found to disperse with time, so extended stopped flow acquisitions were possible without loss of signal strength due to dilution of the sample in the NMR coil. For example, in a 72-h acquisition of a trace sample (data not shown), the first 8-h block of data acquisition was identical to the last 8-h block. This stability also made it possible to interrupt long high-throughput analyses. One automation run of a 96-well plate was suspended in software, and the microcoil probe was removed from the magnet without

disconnecting the transfer line. After using the spectrometer with a different probe for several hours, the microcoil probe was reinstalled, the automation queue was restarted, and analysis of the plate completed without any problems. Sample plugs in Teflon  
5 tubing have been stored for over 1 yr refrigerated with out degradation of the plug nor the analyte.

Sample carryover was below 1%, determined by comparing integrals of solvent peaks between alternating samples of 2% acetone and 4% chloroform, with one wash plug between them. A dye  
10 test showed that carryover to the wash plugs in the inlet capillary was in the range of 20-30 nL (<1%), suggesting most of the 5% sample-to-wash plug carryover observed at the NMR coil occurred in the residual 75- $\mu$ m fused-silica segment of the inlet. The line widths obtained when automatically positioning sample  
15 plugs, evaluated from single-scan spectra, were between 1.0 and 1.6 Hz, close to the routine line width obtained with this probe (1.2 Hz). Importantly, the cost of deuterated solvent consumption was negligible: 0.4 mL of DMSO- $d_6$ /plate at \$2/mL, including 100  $\mu$ L of DMSO- $d_6$  supplied as wash solvent in addition to the 96 3- $\mu$ L  
20 samples.

The efficiency of sample utilization of the SFA-NMR method of this paper was comparable to that attained using capillary isotachopheresis (cITP) (Kautz, 2001; Wolters, 2002b), the most  
sensitive applied NMR method to date. Moreover, SFA-NMR can  
25 relatively quickly load analytes with unknown or zero electrophoretic mobility, which is important for de novo analysis of trace amounts of isolated natural products or drug metabolites. However, most separation and concentration methods cannot be put into practice *in situ* in the remote confined location of the  
30 magnet bore. SFA-NMR permits microseparations and microconcentration to be performed using optimal bench top



techniques and instrumentation as long as a 1- $\mu$ L fraction can be collected for subsequent transfer to the NMR microcoil.

SFA-NMR, as demonstrated above, doubled the throughput, quadrupled the sample efficiency, and reduced deuterated solvent consumption over 20-fold as compared to the commercially supported high-throughput flow NMR methods. Nonetheless, a number of straightforward improvements may still be envisioned. For example, lengthening the transfer line to hold 3 trains without gaps would increase throughput by eliminating the longer sample change time between trains. A larger i.d. sample loop could double the rate of loading the sample loop and could hold more samples in longer trains. Using a 10-port sample loop valve to switch between two loops would eliminate the delay to draw new trains into the sample loop. These and other improvements would increase the throughput of SFA-NMR to over one 96-well plate/h. Using segmented flow to alternately load multiple flow cells (Macnaughtan, 2003; Wolters, 2002c) could additionally increase throughput, reaching essentially continuous NMR data acquisition.

## ADVANTAGES AND OTHER EMBODIMENTS

Because sample efficiency is high and no sample is wasted, trace samples may be analyzed. In fact, use of sample may be 100% efficient, as opposed to 10-30% (in commercial Protasis/MRM microinjection) or 0.1% (filling a 200  $\mu$ m microcoil probe). There is no degradation of sensitivity or resolution if sample plugs as small as 1  $\mu$ L are picked up by the autosampler and transferred into an NMR probe with a 3.5  $\mu$ L flowcell/ 1  $\mu$ L observe volume.

Rapid sample changes are possible along a queue of sample plugs. Conventionally, a sample loaded in the sample loop must be delivered the entire distance to the NMR coil. With the immiscible plug method according to the invention, small sample plugs may be closely spaced, separated by plugs of the immiscible solvent (segmented flow injection). For example, if 1  $\mu$ L sample plugs are

separated by 0.5  $\mu\text{L}$  of immiscible liquids, samples may be changed by moving the queue only 1.5  $\mu\text{L}$ .

Rapid washing of an NMR detection cell is possible. In conventional DI-NMR and FIA-NMR methods, the flow cell must be flushed with several volumes of clean solvent between samples to reduce sample carryover. With the method of the present invention, one sample plug may be followed closely with one or more small plugs of clean solvent to rinse any traces of sample from surfaces or dead volumes of the plumbing. This "train" of sample and rinse plugs may be less than 2  $\mu\text{L}$ , and subsequent samples may follow immediately, in a flow-through injection scheme.

No relaxation time is required after sample injection before NMR analysis can be carried out. Because the sample plug is of uniform concentration, there are not strong concentration gradients within the sample as in the conventional methods. The linewidth of the NMR spectrum is sharp immediately upon arrival of the sample in the NMR coil.

Because samples are maintained in their original volume of 1-2  $\mu\text{L}$ , sample recovery is greatly facilitated. The photograph shown in Fig. 4B is, in fact, of a sample plug after passage through a microcoil NMR probe. With a miscible carrier liquid, analytes disperse over a volume of 5-20  $\mu\text{L}$ . Consequently and in addition, the leading and trailing edges of the resulting analyte zone are not well-defined, and are difficult to detect. With immiscible solvent plugs the sample zone is sharply defined, and can be detected by the physical properties of either solvent or of the sample itself or of the sharp boundary, such as UV or visible absorbance, conductance, viscosity, light scattering, surface tension, or others. A conventional liquid chromatography fraction collector may be used. Alternatively, simply placing a length of Teflon™ tubing on the outlet capillary of the NMR probe collects the sample and wash plugs, which may be discerned by eye.

The diffuse leading and trailing edges of sample plugs in the conventional methods make it difficult to determine the optimal positioning of the sample in the NMR cell. However, with the method of the invention (as shown in Fig. 1B), the leading edge of the sample plug is sharp and easily detected; the plug can be accurately positioned by timing from the arrival of the leading edge.

Samples may be transferred in larger bore capillary tubing, reducing backpressure and consequent need for specialized pumps and related plumbing equipment. Additionally, samples may be transferred over longer distances without loss. High-end NMR spectrometers have larger magnets with larger fringe fields, which may require the sample handler to be as far away as 10 meters. With the present invention there is no disadvantage in sample efficiency or throughput with longer transfer line lengths. Larger capillaries permit fast transfers even over such distances, where 50 micron capillaries would be prohibitive.

While basic "One-Dimensional" NMR spectra are generally acquired from concentrated samples in a few minutes or less, more information or more dilute samples can require NMR acquisition times of several days or more. Using conventional methods, miscible sample plugs can diffuse out of the detection volume over such long periods of time. However, using the method of the invention, the immiscible sample plugs are stable indefinitely. Plugs stored in Teflon™ tubing have remained intact and undiluted for over a year.

Efficient sample transfer by the immiscible plug method according to the invention depends on the relative contact energies of the sample solvent and immiscible carrier solvent with the channel wall. These contact energies can be modified by chemically modifying the channel wall. Figs. 2A-2D show sample plugs of DMSO-d6 (with blue dye) separated by a fluorocarbon liquid (clear), in teflon, fused silica, and perfluoroalkyl

silane-treated fused silica capillaries (FAS-silica). The fused silica capillaries are mechanically more rigid, more suitable for higher pressures, and easier to make high-pressure connections. FAS-silica would enable the existing commercial microcoil probes to use the immiscible plug injection method, because the silica can tolerate the back-pressures generated when driving flow through their 50-micron capillaries.

The use of smaller microcoil probes is enabled using the method of the invention. The current (and only) commercial microcoil NMR probe has an NMR coil detection volume ("active volume," "observe volume,"  $V_{obs}$ ) of 1  $\mu$ l, designed based on the typical size of a capillary LC peak, or of the smallest sample that can be injected using a commercial autosampler, considering the limitations of dilution during transfer. With the present invention, samples of arbitrarily small volume may be efficiently transferred into smaller microcoil probes. In particular, the most sensitive microcoil NMR probes produced to date are wrapped directly on 200/360  $\mu$ m (i.d/o.d.) capillaries and have an observe volume of 30 nL, but sample transfer into the coils is difficult (Kautz, 2001). Using the method of the invention, samples of approximately 30 nL volumes can be efficiently transferred, making these smaller probes, which are three times more sensitive, feasible for routine samples or high-throughput use.

The above work was performed using fluorocarbon FC-43 as the immiscible carrier liquid. Many other immiscible solvent systems, including other fluorocarbon liquids, are available which may be advantageous for their viscosity, immiscibility with unusual analytes or sample solvents, or to match magnetic susceptibility to a particular sample.

Interfacing to capillary separation or concentration microdevices is easy to carry out using the method and system of the invention. A variety of means have been proposed for microanalysis of trace samples by performing separation and

concentration of sub-microliter volumes. Most of these systems are practical or viable in an openly accessible system on the benchtop, or within a specialized device. Most cannot be adapted to practice in the confined and inaccessible volume of an NMR magnet bore, and so cannot be used *in situ* for microcoil NMR as we demonstrated for capillary isotachophoresis. Nor has it previously been feasible to transfer the sub-microliter sample fractions produced by these methods into microcoil NMR probes. The immiscible solvent plug injection method of the invention makes these procedures practical.

Traditional and present methods of flow NMR draw samples from microtiter plates such as 96-well plates with 200  $\mu$ L wells. Arrays of smaller wells such as 384 well plates or 1536-well plates are also in use. A problem in automated sample handling is positioning a needle into the fluid sample volume and withdrawing a small sample completely without drawing any air. With small samples, a significant fraction of small samples must be left in the well, where it is wasted. By the present method, rather than capturing and storing samples in the wells of microtiter plates, samples could be collected at the source of concentration or separation as immiscible plugs in a length of inexpensive teflon tubing filled with the immiscible carrier. The Teflon™ tubing may be easily stored and/or transported to a different laboratory for microcoil NMR analysis or other microfluidic analytical methods.

The present invention also enables a more efficient method of handling small samples in conventional microtiter plates. To draw the entire prepared sample into an autosampler needle without drawing air, an immiscible fluid which is lighter (lower density) than the sample solvent may be added to the sample well together with the prepared sample. This lighter immiscible will float on top of the prepared sample. When the sample is drawn into the needle of the sample handling robot, any excess volume drawn will be the immiscible overlay rather than air, and the sample may be

efficiently transferred into the microcoil NMR or other microfluidic device.

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While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, 35 after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the 40 appended claims and equivalents thereof.